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COMPLETE SPECIFICATION

TREATMENT OF DAIRY PRODUCTS

I/WE, UNION COOPERATIVE AGRICOLE LAITIERE DE LA MANCHE,
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hereby declare the invention, for which I/we pray that a patent may be granted to ~~me~~/us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

Generally speaking, the invention relates to the treatment of whey produced in dairies or casein factories. Its object is more particularly a process permitting the extraction of glycoproteins and/or sialic acid from such a whey. In the present specification the word "whey" means whey produced in dairies or casein factories as well as colostrum.

It is known that dairy whey is a yellowish liquid which, after its fat content has been removed by centrifugation, consists mainly of lactose, proteins and mineral salts.

Treatments for dairy whey have already been suggested, so that it would no longer be a cause of pollution, and to recover the proteins contained therein. Large amounts of whey are produced by dairies and cheese factories, dairy whey being produced from milk after enzyme action, and notably after traditional renneting. Thus, it has been suggested that the proteins should be separated from whey by ultrafiltration.

However, up to now, ultrafiltration has not been used for separating and obtaining certain specific proteins or other compounds which are very useful in themselves.

This is notably the case of sialic acid, also known as N-acetyl neuraminic acid (see, for example, MERCK Index, 7th Edition, p.715). It is known that sialic acid occurs in carbohydrate-protein complexes of animal origin. In actual fact, this compound is at present prepared either from natural raw materials such as the sub-maxillary glands of bovines, or by syntheses.

As a bibliographical reference in this connection may be mentioned the article by M.W. WHITEHOUSE and

F. ZILLIKEN "Isolation and Determination of Neuraminic (sialic) acids" p.199 to 220 in "Methods of Biochemical analysis, Volume VII (1960) Interscience, John Wiley Sons.

These known processes for the preparation of sialic acid are extremely costly and this high cost of production is passed on when the product is put on the market.

As bibliographical references to certain applications of sialic acid, and particularly of NANA, the following articles may be mentioned :
"Coagulation of milk with rennet : Scientific and technical aspects".

- GARNIER, MOCQUOT, RIBADEAU-DUMAS, MAUBOIS- Ann. de Nutrition Alimentaire, 1968,22 B 495 - B 552.

- SVENNERHOLM. L. Acta. Soc. Med. Upsaliensis, 61,75 (1956) Arkiv. Kemi., 10,577 (1956).

- WARREN L. J. Biol. Chem, 233, 1971 (1959)

- WERNER I. and L. ODIN, Acte Soc. Upsaliensis 57, 230 (1952)

- AMINOFF, D (1961) BIOCHEM J. 81,384

- "The Sensitivity of the Neuraminosidic Linkage in Mucosubstances towards Acid and towards Neuraminidase Gibbons". Biochemistry Journal (1963) 89, 380.

- "Structure studies on the Myxovirus Hemagglutination Inhibitor of Human Erythrocytes" Ralph H. KATHAN and Richard J. WINZLER. Journal of Biological chemistry (1963) Vol.238 N° 1, p.21.

- "Studies on the Neuraminidase of Influenza

virus II additional properties of the enzymes from the Asian and PR 8 Strains. Max E. RAFELSON, J.R. Michael SOMMER and Wannie W. WILSON J.R. Archives of Biochemistry and Biophysics 103 (1963) 424-430.

Other possible uses of sialic acid are given in the literature relating to this compound.

In another connection, it is advantageous to be able to obtain glycoproteins owing to the possibility of using them in cosmetic compositions.

An object of the present invention is a process for the treatment of whey produced by dairies or casein factories which makes it possible to obtain sialic acid very cheaply, and more specifically N-acetyl neuraminic acid (abbreviated to NANA), jointly with glycopeptides and a protein fraction consisting of glycoproteins.

The invention therefore relates to a process for the production of sialic acid and/or glycoproteins from dairy or casein factory whey, with ultrafiltration of same, characterized by the steps of :

(a) thermal flocculation of the proteins of cheese factory whey, other than sialoglycoproteins, resulting in a first precipitate of flocculated proteins and a first supernatant, which is separated and recovered.

(b) ultrafiltration of the first supernatant on membranes having a cut-off in the range of ~~about~~ 1000 to ~~about~~ 15,000 in molecular weight providing a retentate containing glycoproteins and sialic acid.

(c) hydrolysis of said retentate.

(d) subsequent ~~treatment~~ processing of said hydrolysate

to extract the sialic acid therefrom.

According to one embodiment of the process, the hydrolysed retentate obtained in step (c) provides a second precipitate and a second supernatant which is separated and recovered. The treatment (d) of said second supernatant for the extraction of the sialic acid contained therein consists in known operations essentially involving the steps of neutralization, flowing the last supernatant over cationic resin, fixing the sialic acid by passing it over anionic resin, elution of the acid so fixed and the recovery of an extremely pure sialic acid which may be freeze-dried.

As raw material, there is used in the process of the invention a milk produced by any ruminant (cow, goat, ewe, buffalo or the like), for example cows or ewes milk having undergone enzymatic action, such as renneting, providing a whey known as cheese factory whey. Said liquid whey can be obtained by the addition of water to a powdered whey. It should also be noted that, as a variant, colostrum may be used as raw material in the process.

The first step (step "a") of the process of the invention consists in a selective denaturation of the soluble proteins by thermal floculation at a temperature and for a period of time sufficient to obtain such a floculation. The albumines and globulines are precipitated and the protease peptones, which are glycoproteins, are retained in the supernatant. It is advisable to heat to relatively high temperatures,

although they should not rise above 100°C to avoid denaturation of the glycoproteins. With lower temperatures a longer period of heating is necessary. Conditions which have been found suitable in practice and which are, moreover, usual in this type of technique, consist of heating at 95°C for 30 minutes. The flocculated proteins obtained at the end of step "a" are separated by any known means, such as centrifugation, and then recovered. The supernatant is recovered for use in the subsequent steps of the process.

In the next step "b", the supernatant is subjected to ultrafiltration by being passed over membranes having a cut-off in the range of about 1000 to 15,000, expressed by molecular weight. Membranes suited to use are of all known types, organic, inorganic, and even ceramic or metallic ones insofar as they satisfy the requirements for cut-off which have been given hereinabove.

As an example, it is possible to use the membranes put on the market by the firm RHONE POULENC under the name of IRIS, for instance, an IRIS 3042 membrane which has a cut-off of about 15,000 in the ultrafiltration modules also manufactured by the said firm. It is also possible to use the membranes sold by the firm AMICON (USA) under the name of DIAFLO, such as the membranes DIAFLO PM 10 and U 1 (cut-off : 10,000) and DIAFLO UM2 (cut-off : 1000). If so required, all the requisite information on the nature and mode of use of the aforesaid membranes can be found in the technical leaflets put out by the manufacturer.

The conditions of ultrafiltration can be understood by a man skilled in the art. It is preferable to circulate the liquid through an ultrafiltration module to contact it with the membrane at a temperature of approximately ambiant temperature andunder a certain pressure, for example, at 3 bars. The product circulating over the membrane can be recycled several times until a retentate is obtained having the desired glycoproteins and NANA content.

Said retentate can be concentrated to obtain a syrup containing glycoproteins. For the extraction of sialic acid, the retentate is subjected to step "c" which consists of an hydrolysis. This can be an acid, basic or enzymatic hydrolysis. Acid hydrolysis conditions are, however, preferred. In order to increase the speed of hydrolysis, it is advantageous to work at a relatively high temperature, but this should be lower than 98°C, about 90°C for example. The acidity of the hydrolysis agent used should not generally exceed 0.5N . It is advantageous to use sulphuric acid, for example, 0.1 N sulphuric acid. This supplies sulphate ions which are subsequently easily separated. Hydrochloric acid is less suitable as it supplies chloride ions which are difficult to remove later on. In accordance with the conventional method for facilitating the appearance of the precipitate produced by hydrolysis, the reaction medium obtained from hydrolysis is cooled, for example to about 4°C which is the temperature of a refrigerator. It is then easier to separate the precipitate from the

supernatant by any known means, notably by centrifugation. The precipitate produced during hydrolysis is removed and the supernatant is recovered to be subjected in a step "d" to a further treatment permitting the extraction of sialic acid, and more precisely NANA.

At this stage of the treatment of dairy or casein factory whey, the invention makes use of the known technique for the production of sialic acid. This starts with the neutralization of the supernatant with a view to precipitating in the form of salts the free acid ions still present in the supernatant. This operation is advantageously effected by the addition of excess ~~baryum~~ barium hydroxide to precipitate the sulphate ions if hydrolysis was effected with sulphuric acid. An excess of ~~baryum~~ barium ions is used until an approximately neutral pH is obtained.

The precipitated salts formed such as ~~baryum~~ barium sulphate, are then removed and the supernatant is retained. This is optionally concentrated before being flowed through resin columns. A first flow through is effected on cationic resin in order to demineralize the supernatant. Resins available on the market under the name of "DOWEX", such as type AG 50 WX 8 H+ are, for examples, used. After passing across cationic resin, the product is flowed through a column of anionic resin in order to fix the NANA. The resin sold under the name of "DOWEX" type AG 1 X 8 formate is suitable for this purpose. The NANA is then obtained from the said anionic resin after washing the colu

distilled water and by elution, notably with formic acid if an anionic resin in the formate form, such as 0.3M formic acid, has previously been used.

A solution is finally obtained which, after freeze-drying, results in an extremely pure NANA powder.

The steps making up treatment "d" can undergo variations. For example, after neutralization, separation of the ~~baryum~~ barium sulphate and clarification of the supernatant, this last can be dried. The powder obtained is then subjected to solvent extraction, that is to say, it is mixed with a solvent or solvents in which NANA is soluble, such as ethanol, or an acetone-water mixture. The NANA extract is then isolated after elimination of the solvent.

According to a preferred embodiment of the process, it is also possible to induce preliminary precipitation of the free acid ions after hydrolysis, i.e. before removal of the desialised proteins. In order to carry this out the solution, the temperature of which after hydrolysis is in the range of 50°C to 80°C, and preferably between 70°C and 80°C, for example, approximately 80°C, is neutralized at pH 7 - 7.5.

As has already been stated, hydrolysis can be effected by acid, basic, or enzymatic treatment. Acid hydrolysis is preferred, sulphuric acid being advantageously used. In this last hypothesis, neutralization is effected with an excess of ~~barium~~ barium hydroxide or barium hydrate.

After clarification adapted to remove the heavy ~~baryum~~ barium sulphate precipitate, the solution is again subjected to ultrafiltration by being flowed across membranes having a cut-off in the range of about 500 to about 15,000.

The ultrafiltrate obtained after the second ultrafiltration can be treated as in step (d) described hereinabove to obtain extremely pure sialic acid, that is to say, by being flowed over cationic and then over anionic resins.

According to a particular embodiment of the invention, it is also possible to obtain less pure sialic acid by drying the solution obtained after it has passed over the cationic resin.

The sialic acid thus isolated which is less costly to produce than the pure acid, can be particularly useful for certain applications, such as for use in cosmetics, where absolute purity is not required.

The purity of the sialic acid thus obtained lies between about 60% and about 90% and can be, for example, of approximately 70%. The man of the art will understand that it is possible to vary this degree of purity as a function of at least three parameters such as :

- the degree of purification of the glycoproteins by the first ultrafiltration,
- the quality of hydrolysis,
- the quality of the membrane used for the second ultrafiltration, the lower the cut-off the [illegible]

the ultrafiltrate.

The process of the invention is illustrated by the appended tables, which show in two practical modes of embidiment the succession of steps of the process and the circulation of materials. These tables clearly illustrate the process. It will be noted that, as the raw material which may be used, there is mentioned either dairy or casein factory whey B, or whey B reconstituted from powdered whey B' by the addition of water, or colostrum A'. The various fractions obtained by the process consist of floculated proteins separated after floculation 1, of glycoproteins which may be obtained by concentration of the retentate (5) obtained from ultrafiltration (4), and, finally, the NANA isolated either according to the diagram of table I by hydrolysis (6) of the retentate, cooling to about 4°C (7) neutralization of the supernatant (9), and flowing said clarified supernatent over cationic resins (14) and anionic resins (15) and freeze-drying (13), or, according to table II, by hydrolysis of the retentate, cooling to about 80°C, ultrafiltration and flowing the ultrafiltrate over cationic resin to recover NANA having about 40% purity, or flowing over anionic resin according to the diagram of table II.

The invention will now be illustrated, while in no way being limited, by the following examples :

EXAMPLE 1

The traditional technique was used to rennet 1000liters of cows milk which yielded 90liters of liquid whey for use as raw material for the process.

The 900 liters of liquid whey were heated at 95°C for 30 minutes preparatory to the flocculation of proteins other than sialoglycoproteins, the proteins thus flocculated being centrifuged and recovered. The supernatant resulting from the flocculation representing a volume equal to 96% of the initial volume and containing 173 grammes of NANA was recovered.

Said supernatent was placed in an ultrafiltration module equipped with a membrane having a cut-off of 3000; the pressure in the ultrafiltration module was approximately 3 bars. A retentate was thus obtained containing all the glycoproteins and 90 grammes of NANA. By concentrating said retentate a syrup of glycoproteins was obtained which could be used as it was.

In order to extract the sialic acid therefrom the retentate, preferably after concentration, is subjected to acid hydrolysis by the addition of 0.1N sulphuric acid, hydrolysis conditions being maintained at 90°C for one hour. The hydrolysis reaction medium was cooled to approximately 4°C, the temperature of a refrigerator in order to facilitate precipitation. It was then easy to separate the hydrolysis precipitate, which was rejected, from the supernatant which was kept, and which contained 80 grammes of NANA. Neutralization was then effected by treating the supernatant with a saturated baryum hydroxide solution until a pH 7 was obtained, with precipitation of ~~baryum~~ barium sulphate. The solution was clarified, the ~~baryum~~ barium sulphate formed being eliminated. The supernatant containing 80 grammes

of NANA was retained and concentrated to reduce its volume 4 to 6 fold by means of a vacuum rotary evaporator heated to 45°C and operating at a pressure of 20 to 30 mg Hg. The supernatant so concentrated was flowed through a column packed with DOWEX cationic resin, type AG 50 WX 8 H+ for demineralisation. At the outlet of the cationic column the product was flowed through a column of DOWEX anionic resin, type AG 1 X 2 formate to fix the NANA. The column containing the anionic resin was then washed with double-distilled water and the NANA was eluted with 0.3M formic acid. 70% of the NANA fixed was thus recovered; after freeze-drying of the formic solution, 45 grammes of extremely pure NANA was obtained.

EXAMPLE 2

Working under identical conditions to those described in example 1, but starting with 1000 liters of ewes milk, substantially identical results were obtained.

EXAMPLE 3

This operation was carried out under the same conditions as in example 1, but starting with a liquid whey obtained by the regeneration of powdered whey. For this purpose, 50 kg of powdered whey was used diluted to obtain 900 liters of liquid lactoserum.

EXAMPLE 4

1000 liters of liquid whey were floculated by heating; the clear filtrate obtained by centrifuging and filtration was subjected to ultrafiltration on a RP membrane (IRIS 3042). The retentate obtained (10 liter

contained 40 g/l of glycoproteins which could be extracted by drying and freeze-drying.

The retentate was hydrolysed with 0.025 N H_2SO_4 at 90°C for 25 minutes then neutralized at 30°C by excess ~~baryum~~ barium oxide to pH 7-7.5.

After clarification, the solution freed of its ~~baryum~~ barium sulphate precipitate was subjected to ultrafiltration on a membrane with a cut-off of 5000.

The ultrafiltrate obtained is concentrated and flowed over cationic resin. The solution obtained can be dried to obtain 30g of sialic acid (NANA) (purity : 70%) or flowed over anionic resin, eluted and freeze-dried thus making it possible to obtain 35g of extremely pure NANA.

WHAT WE CLAIM IS:

1. A process for producing glycoproteins and/or sialic acid from dairy or casein factory whey, with ultrafiltration thereof, characterized by the steps of:

(a) thermal flocculation of whey proteins, other than the sialoglycoproteins, providing a first precipitate of flocculated proteins and a first supernatant, which is separated and recovered;

(b) ultrafiltration of the first supernatant on membranes having a cut-off of between 1,000 and 15,000 in molecular weight, providing a retentate containing glycoproteins and sialic acid;

(c) hydrolysis of said retentate;

(d) subsequent processing of said hydrolysate to extract the sialic acid therefrom.

2. A process for producing glycoproteins and/or sialic acid from colostrum, with ultrafiltration thereof, characterized by the steps of:

(a) thermal flocculation of whey proteins, other than the sialoglycoproteins, providing a first precipitate of flocculated proteins and a first supernatant, which is separated and recovered;

(b) ultrafiltration of the first supernatant on membranes having a cut-off of between 1,000 and 15,000 in molecular weight, providing a retentate containing glycoproteins and sialic acid;

(c) hydrolysis of said retentate;

(d) subsequent processing of said hydrolysate to extract the sialic acid therefrom.

3. A process according to either of claim 1 or 2, characterized in that, during step (a) flocculation

of proteins other than the sialoglycoproteins of dairy whey is effected by thermal treatment at a temperature and for a period of time sufficient to obtain such a flocculation.

4. A process according to claim 3, characterized in that flocculation is effected at temperatures lower than 100°C.

5. A process according to either of claim 3 or 4, characterized in that the thermal treatment is effected at about 95°C for about 30 minutes.

6. A process according to any of claims 1 to 5, characterized in that, in step (b) the liquid supernatant is circulated in an ultrafiltration module to contact it with the membrane at a temperature approximately that of ambient temperature and under a pressure of 3 bars, it being possible for the product circulating over the membrane to be recycled several times until a retentate is obtained having the desired glycoprotein and ~~NANA~~ sialic acid contents.

7. A process according to any one of claims 1 to 6, characterized in that the retentate obtained in step (b) is concentrated and a glycoprotein syrup is obtained.

8. A process according to any one of claims 1 to 6, characterized ~~in that~~ by an additional extraction sialic acid ~~is extracted~~ from the retentate obtained in step (b).

9. A process according to claim 8, characterized in that, during step (c) the ultrafiltration retentate of step (b) is hydrolysed, hydrolysis being effected by acid, base or enzyme.

10. A process according to either of claims 8 or 9, characterized in that acid hydrolysis is effected,

with acid, preferably sulphuric acid, at a concentration weaker than 0.5N.

11. A process according to any of claims 8 to 10, characterized in that hydrolysis is effected at a temperature no higher than 98°C.

12. A process according to claim 1, characterized in that the said hydrolysed retentate obtained in step (c) is cooled to about 4°C and provides a second precipitate and a second supernatant which is separated and recovered and in that processing (d) of said second supernatant comprises essentially the steps of neutralization, passing the last supernatant over cationic resin, fixing the sialic acid by passing over anionic resin, elution of the acid so fixed and recovery of extremely pure sialic acid by freeze-drying of the sialic acid solution.

13. A process according to any of claims 1, 10 or 11, characterized in that, during processing (d), the second supernatant obtained after hydrolysis step (c) is neutralized to precipitate the free acid ions in the form of salts, preferably by the addition of excess barium hydroxide, to precipitate the sulphate ions if hydrolysis was effected with sulphuric acid.

14. A process according to claim 1, characterized in that the said hydrolysed retentate obtained in step (c) is neutralized at a temperature of between 50°C and 80°C, and in that the clarified solution is subjected to ultrafiltration on membranes having a cut-off of between 500 and 15,000 and in that the sialic acid is extracted from the ultrafiltrate obtained.

15. A process according to claim 12, characterized in that, during processing (d) the neutralized and

second supernatant optionally concentrated, is flowed over a cationic resin, then over an anionic resin, after which the column of anionic resin is washed and elution is effected to recover the N-acetyl neuraminic acid (NANA) or sialic acid fixed to the said resin, elution being effected with the acid having the same anion as the anion used in the anionic resin.

16. A process according to claim 14, characterized in that during processing (d) the neutralized second ultrafiltrate optionally concentrated, is flowed over a cationic resin, then over an anionic resin, after which the column of anionic resin is washed and elution is effected to recover the N-acetyl neuraminic acid (NANA) or sialic acid fixed to the said resin, elution being effected with the acid having the same anion as the anion used in the anionic resin.

17. A process according to claim 1 or 2, characterized in that, during processing (d), the supernatant is neutralized and clarified and is then dried and the powder obtained is put into intimate contact with a solvent or mixture of solvents in which NANA is soluble, and the NANA is then isolated after elimination of the solvent.

18. A process according to claim 14, characterized in that sialic acid is extracted by passing said ultrafiltrate over a cationic resin and the solution so obtained is dried to give sialic acid.

19. N-acetyl neuraminic acid (NANA) or sialic acid obtained by the process according to any of claims 1 or 11, 14, 16 or 17 notably freeze-dried and substantially pure.

20. N-acetyl neuraminic acid (NANA) or sialic acid obtained by the process according to any one of claims 12, 13 or 15 notably freeze-dried and extremely pure.

21. The protein fractions, notably glycoproteins, obtained by the process according to any of claims 1 to 7.

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by their attorneys
BALDWIN SON & CAREY

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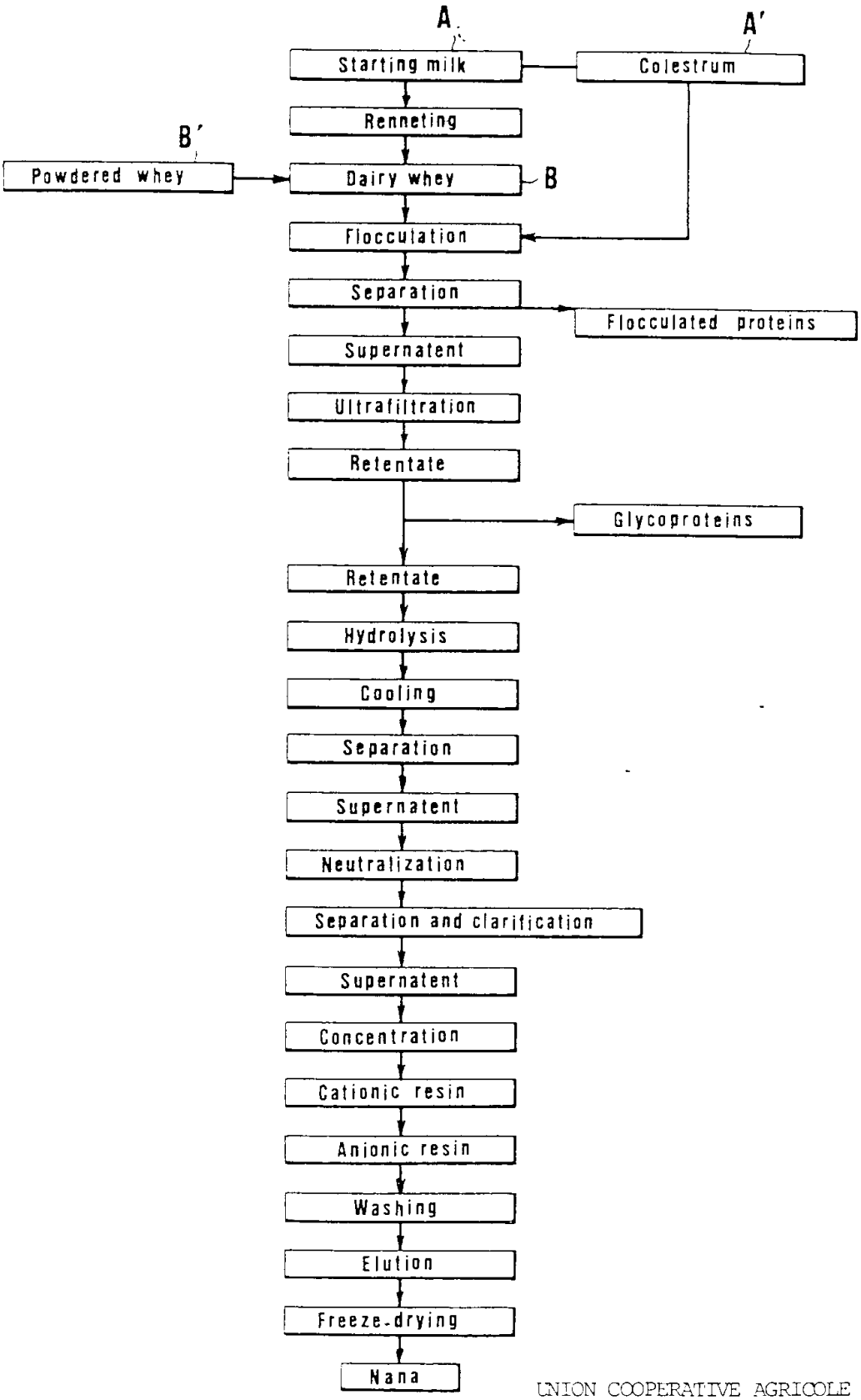

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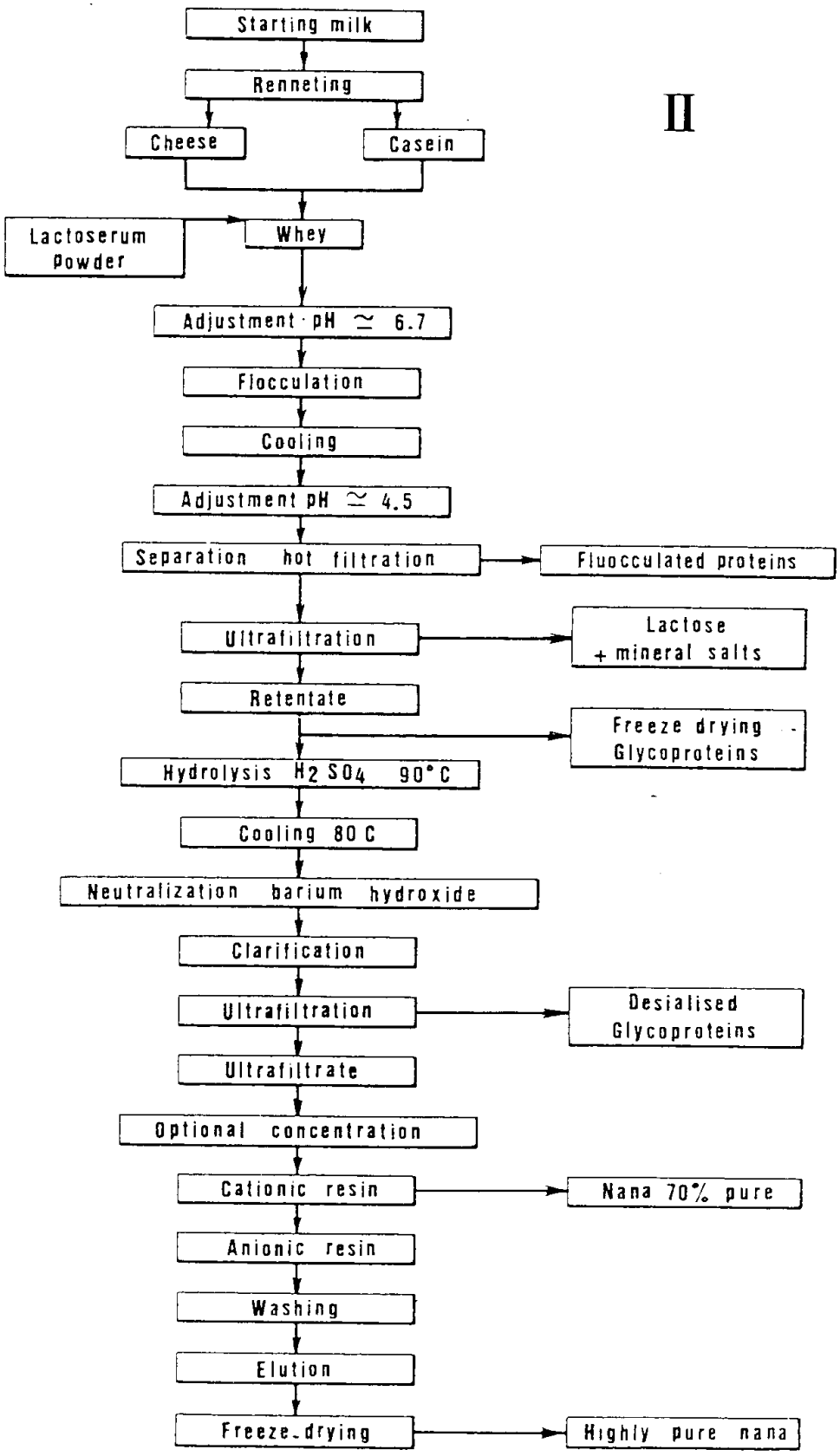

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